

**(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)**

**(19) World Intellectual Property Organization**  
International Bureau



**(43) International Publication Date**  
29 November 2001 (29.11.2001)

**PCT**

**(10) International Publication Number**  
**WO 01/90390 A1**

**(51) International Patent Classification<sup>7</sup>:** **C12N 15/86**, (74) **Agent:** **GILL JENNINGS & EVERY**; Broadgate House, A61K 48/00, A61P 9/10 7 Eldon Street, London EC2M 7LH (GB).

**(21) International Application Number:** PCT/GB01/02383

**(22) International Filing Date:** 29 May 2001 (29.05.2001)

**(25) Filing Language:** English

**(26) Publication Language:** English

**(30) Priority Data:**  
0012997.3 26 May 2000 (26.05.2000) GB

**(71) Applicant** (for all designated States except US): **ARK THERAPEUTICS LIMITED** [GB/GB]; 6 Warren Mews, London W1T 6AR (GB).

**(72) Inventors; and**

**(75) Inventors/Applicants** (for US only): **YLA-HERT-TUALA, Seppo** [FI/FI]; A.I. Virtanen Institute, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio (FI). **AIRENNE, Kari, J.** [FI/FI]; A.I. Virtanen Institute, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio (FI).

**(81) Designated States (national):** AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

**(84) Designated States (regional):** ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



**WO 01/90390 A1**

**(54) Title:** USE OF BACULOVIRUS VECTORS IN GENE THERAPY

**(57) Abstract:** In a method for the delivery of a gene product, the gene is provided in a baculovirus vector and the vector is applied to a body compartment free, or usually free of blood. In a device for the periadventitial delivery of a gene product, the gene is provided in a baculovirus vector from which the gene may be expressed.

## USE OF BACULOVIRUS VECTORS IN GENE THERAPY

Field of the Invention

This invention relates to gene delivery using a viral vector.

Background of the Invention

5        Efficient gene transfer would be a beneficial tool for the treatment of vascular diseases, such as post-angioplasty restenosis, post-bypass atherosclerosis, peripheral atherosclerotic disease, stenosis of vascular prosthesis anastomoses, and thrombus formation. Various techniques have been developed for this purpose; see, for example, Yla-Herttula *et al*, *J. Clin. Invest.* 95:2692-8 (1995), and Laitinen *et al*, *Hum. Gene. Ther.* 8:1645-50 (1997).

10      WO-A-98/20027 discloses a periadventitial collar that can be used for arterial gene transfer during vascular surgery. However, there is a continuous need for more facile and efficient gene transfer vectors. Only a temporary expression of the transgene may be required to achieve a beneficial biological effect in cardiovascular applications; see Yla-Herttula *et al*, *Lancet* 355:213-222 (2000).

15      Baculoviruses have long been used as biopesticides and as tools for efficient recombinant protein production in insect cells. They are generally regarded as safe, due to the naturally high species specificity and because they are not known to propagate in any non-invertebrate host. Although the virions have been shown to 20 enter certain cell lines derived from vertebrate species, no evidence of viral gene expression has been detected using natural viruses. However, the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), containing an appropriate eukaryotic promoter, is able to transfer and express target genes efficiently in several mammalian cell types; see, for example, Hofmann *et al*, *PNAS USA* 92:10099-10103 25 (1995). In addition, Barsoum *et al*, *Hum. Gene. Ther.* 8:2011-8 (1997), has reported that baculovirus having the vesicular stomatitis virus C glycoprotein in its envelope significantly increases the efficiency of transduction of human hepatoma cell lines and broadens the range of mammalian cell types that can be transduced by baculoviruses. Stable transduction of mammalian cells by baculoviruses has been achieved by either 30 including an expression cassette encoding a dominant selectable marker into baculovirus genome or by using hybrid baculovirus-adeno-associated virus vector; see Condreay *et al*, *PNAS USA* 96:127-132 (1999), and Palombo *et al*, *J. Virol.* 72:5025-34 (1998).

35      Sandig *et al*, *Hum. Gene Ther.* 7:1937-45 (1996), reported unsuccessful attempts to use baculoviruses for *in vivo* gene delivery in mice and rats by systemic or

intraportal application as well as by direct injection into the liver parenchyma. One reason for this is presumably the inactivation of baculoviruses by the classical pathway of serum complement system.

WO-A-00/05394 discloses baculovirus vectors and their use for gene transfer  
5 to the nerve cells of vertebrates.

#### Summary of the Invention

It has now been found that the inactivation of baculoviruses can be avoided. In particular, it has been shown that baculoviruses are able to mediate periadventitial gene transfer to rabbit carotid arteries with an efficiency comparable to adenoviruses.

10 The ease of manipulation and rapid construction of recombinant baculoviruses, their lack of cytotoxicity in mammalian cells even at a high multiplicity of infection, their inherent incapability to replicate in mammalian cells, and their large capacity for the insertion of foreign sequences, make baculoviruses very suitable tools for *in vivo* gene therapy.

15 This invention is able to use the advantageous properties of baculoviruses, in a suitable vector, from which the gene is expressed, if administered (*in* or *ex vivo*) to a body site at which there is no blood, or which is essentially free of blood. Thus, periadventitial or, more specifically, collar-mediated local gene delivery allows gene transfer essentially in the absence of serum, thus avoiding deleterious effects of serum 20 components. The novel method also avoids two other major problems encountered in systemic gene delivery, i.e. a rapid redistribution of the virus from the injection site and a drop in the local concentration of the virus.

#### Description of the Invention

Suitable delivery systems, active materials, formulations, dosages etc, are  
25 illustrated in WO-A-98/20027 and also WO-A-99/55315 (the contents of which are incorporated herein by reference). Thus, by way of example only, the delivery vehicle may be a collar or wrap. By comparison with those publications, the vector for gene delivery is a baculovirus.

Baculoviruses are of course known, and the skilled person will be able to  
30 construct any suitable vector for use in this invention. It will also be evident that the broad knowledge of baculovirus biology and AcMNPV genome will aid engineering of the improved second-generation viruses for gene transfer applications. The ease of construction, and capacity to accept large foreign DNA-fragments (>20 kbp), allows the development of baculoviruses having enlarged or targeted cell tropism along with more  
35 stable, temporal and cell type-specific control of transgene expression. A recombinant

baculovirus for use in the invention may be formulated into a medicament for therapeutic use, in known manner.

Routes and sites of administration for the invention include intra-ocular application, intra-articular application, superficial intra-dermal application, ureters, 5 bladder, Fallopian tubes, gall bladder, spinal cord, cerebrospinal fluid compartment, pleural cavity and intraperitoneal cavity. Sites that have been used (see the Examples) are arteries, brain and skeletal muscle, including, by way of example, myocytes, satellite cells and regenerating myoblasts. Gene delivery may be done *via* direct injection or various types of catheters.

10 If appropriate, body parts can be made "bloodless" during surgery. This technique is often used in leg or arm surgery by putting tight pressure around arm or thigh, thus preventing blood flow. The body part may then be perfused with saline to remove blood, and baculovirus transfection can then be done.

15 The invention can be used for the delivery of an agonist of a VEGF receptor, e.g. described in more detail in WO-A-98/20027. Further, by suitable choice of the gene, it may be used in the treatment of cancer, e.g. in the brain.

A further aspect of the invention relates to transplant organs and vessels which can be perfused with saline *ex vivo* and subjected to *ex vivo* baculovirus injection.

The following experimental work illustrates the invention.

20 **Example 1**

***Preparation of recombinant baculoviruses***

Viruses were constructed by using the transfer vector pFASTBac1 (pFB) (Gibco BRL, Life Technologies, Gaithersburg, MD, USA). A nuclear targeted  $\beta$ -galactosidase ( $\beta_{nt}$ -Gal) cassette with a cytomegalovirus (CMV) promoter was inserted into the *Stu* site 25 of pFB in reverse orientation with respect to the polyhedrin promoter, generating plasmid pFBCMV- $\beta_{nt}$ .

Recombinant viruses were generated by using a BacTo-Bac<sup>TM</sup> Baculovirus Expression System (Gibco BRL). Viruses were amplified in *Spodoptera frugiperda* 9. (Sf9) suspension cultures (SF-900 medium, Gibco BRL) for 3 days using cell density 30 of  $2 \times 10^6$  cells/ml. For 50 ml of culture, 200  $\mu$ l of primary transfection supernatant was used as an inoculum. To obtain 1 litre of amplified virus, 2 ml of amplified virus stock was used as an inoculum. The cell culture medium was centrifuged at 16,000 g for 20 min at room temperature to remove cell debris. The clarified supernatant was transferred to ultracentrifuge tubes underlaid with 1.5 ml of 25% sucrose in phosphate-35 buffered saline (PBS) and the viruses were concentrated by centrifugation (120,000 g,

4°C, 1.5 h). The virus pellets were resuspended into 35 ml of ice-cold PBS, transferred into ultracentrifuge tubes containing 3 ml of 25% sucrose in PBS and centrifuged as above. Final virus pellet was resuspended into 10 ml of cold PBS, filtered through the 0.45 µm filter and kept at 4°C protected from light for further use. Virus titer was 5 determined by a plaque assay on Sf9 cells. Virus preparations were analyzed for lipopolysaccharide and bacteriological contaminants.

#### ***Preparation of recombinant adenoviruses***

Nuclear-targeted *LacZ* encoding adenoviruses (pCMVnls/acZAd5) were constructed and prepared as described by Laitinen *et al*, *supra*. Virus preparations 10 were analyzed for replication-competent viruses, lipopolysaccharide and bacteriological contaminants as described by Laitinen *et al*, *Hum. Gene Ther.* 9:1481-6 (1998).

#### ***Gene transfer in vitro***

Rabbit aortic cells (RAASMC; Yla-Herttula *et al* (1995), *supra*) and human carcinoma/ endothelial cell-like ECV-304 cells (ATTC CRL-1998) were plated at the 15 density of 10,000 cells per well (Falcon Culture Slide, Becton Dickinson, Meylan, France). Cells were allowed to attach for 3 h before transduction in serum-free medium (DMEM, 100 units/ml of penicillin and 100 µg/ml of streptomycin, Gibco BRL). Viruses were added to medium at MOIs of 200 or 1000, and cells were incubated for 90 min at 37°C. After transduction, growth medium containing 10% fetal bovine serum was 20 added either with or without 10 mM n-butyric acid (Sigma, St. Louis, MD, USA). After 18 h incubation, the media were removed and cells were washed three times with PBS. Cells were fixed with 1.25% glutaraldehyde for 15 min and washed three times with PBS. X-Gal (MBI Fermentas, Lithuania) staining solution (1 mg/ml, 2 mM MgCl<sub>2</sub>, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 1 x PBS) was added to the cells and incubated for 3 h at 25 37°C. Cells were then washed with PBS and further fixed with 4% paraformaldehyde (PFA) for 10 min. After washing with PBS, cells were counter-stained with Mayers Carmalum for 5 min. Blue X-Gal positive cells were counted and the transduction efficiencies were expressed as the percentage of positive cells of the total number of cells.

#### ***ONPG assay***

*lacZ*-encoded β-galactosidase activity in transduced RAASMC cells was measured using a colorimetric substrate, o-nitrophenyl β-D-galactopyranoside (ONPG, Sigma), as described by Ruponen *et al*, *Biochim. Biophys. Acta* 1415:331-41 (1999).

***In vitro toxicity assay***

Cells were plated in 96-well plates at a density of 20,000 cells per well in 100 µL of growth medium consisting of DMEM with 10% fetal bovine serum and antibiotics (100 units/mL of penicillin and 100 µg/mL of streptomycin). Virus transductions were 5 performed as for the transduction efficiency assay and cells were incubated for 48 h at 37°C. Growth medium was removed and cells were washed with PBS. Serum-free DMEM without phenol red, containing MTT solution (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide, 5 mg/mL, final concentration 0.3 mg/ml) was then added and cells were incubated for 2 h. To dissolve the dark blue formazan crystals, MTT 10 solution was removed, 150 µl of 1 M DMSO was added to each well and mixed thoroughly. Absorbance was measured at 570 nm. Survival percentage was calculated as compared with absorbance of the no virus- or no butyrate wells (100% survival).

***Animal experiments***

Male New Zealand White (NZW) rabbits ( $n = 12$ ; 2.8-3.7 kg) were used. 15 Fentanyl-fluanisone (0.3 ml/kg, s.c.; Janssen Pharmaceutica, Beerse, Belgium) and midazolam (1.5 mg/kg, i.m., Roche, Basel, Switzerland) were used for anesthesia. Left and right carotid arteries were exposed using midline neck incision. The artery was carefully separated from the surrounding tissue and a 3 cm long silastic collar (see Laitinen *et al* (1997), *supra*) was positioned around it. Rabbits were re-anesthetized for 20 gene transfer, which was performed 5 days after the installation of the collar exactly in the same way as described by Laitinen *et al* (1997), *supra*, comparing the transfection efficiency of plasmid/liposomes, pseudotyped retroviruses and adenoviruses. The collars were opened and filled with 500 µl of the gene transfer solution containing 1 x 10<sup>9</sup> pfu of adenovirus or baculovirus. In each animal, the left carotid artery was used 25 for adenovirus and the right carotid artery for baculovirus treatment. Four rabbits were killed 3, 7 and 14 days after the gene transfer and arteries were removed for histological analyses. All animal procedures were approved.

***Histological analysis***

Collared arteries were divided into three equal parts: the proximal third was 30 immersion-fixed in 4% PFA/15% sucrose (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) overnight and embedded in paraffin. The medial part was immersion-fixed in 4% PFA/PBS (pH 7.4) for 30 min, rinsed in PBS (pH 7.2) and embedded in OCT compound (Miles Scientific, Naperville, IL, USA). The distal part was snap-frozen in liquid nitrogen and stored at -70°C for mRNA isolation and reverse transcriptase polymerase chain 35 reaction (RT-PCR).

Randomly selected frozen sections (10  $\mu$ m) from each rabbit were stained with X-Gal (MBI Fermentas) for 18 h to identify  $\beta$ -galactosidase positive cells. Gene transfer efficiency was calculated as X-Gal positive cells per mm<sup>2</sup> of adventitia or whole artery wall in eight randomly selected sections by two independent observers. Mean values 5  $\pm$  standard error of mean (SEM) of the results are reported. Paraffin sections were used for immunocytochemical detection of endothelium (CD-31; 1:50 dilution; Dako, Hamburg, Germany), macrophages (RAM-11; 1:100 dilution; Dako), smooth muscle cells (HHF-35; 1:50 dilution; Dako), and T cells (MCA-805; 1:100 dilution; Dako) as described by Yla-Herttula (1995), *supra*. Controls for the immunostainings included 10 sections where the first antibody was omitted and sections incubated with class and species matched immunoglobulins. Morphometry and image analysis were done using hematoxylin-eosin-stained paraffin sections and Image-Pro Plus<sup>TM</sup> software with Olympus AX70 microscope (Olympus Optical, Japan).

#### **RT-PCR**

15 Total RNA was extracted from transduced carotid artery segments using Trizol Reagent (Gibco-BRL) and treated with excess RQ1 RNase-free DNase (Promega, Madison, WI, USA). M-MuLV reverse transcriptase (MBI Fermentas) was used for cDNA synthesis. Primers (20 pmol each) for *lacZ* were designed to distinguish between 20 endogenous and transduced genes by selecting the 5' primers from the CMV promoter and the 3' primers from the coding region. Dynazyme polymerase (Finnzymes, Espoo, Finland) was used for amplification.

25 For *lacZ* amplification, the primers were SEQ ID NO:1 for adenovirus (A) and SEQ ID NO:2 for baculovirus (B) as forward primers, and SEQ ID NO:3 for both as a reverse primer. Hot start (95°C 5 min; 58°C 3 min) was followed by 39 cycles, each consisting of 95°C 1 min, 58°C 2 min, 72°C 3 min with the final extension of 10 min at 72°C. 5  $\mu$ l of the first PCR product was used for the second PCR with forward primers SEQ ID NO:4 (A) and SEQ ID NO:5 (B). The reverse primer for both was SEQ ID NO:6. The first PCR cycle was 95°C for 5 min followed by 20 cycles as in the first PCR.

#### **Gene transfer *in vitro***

30 In order to test the baculovirus stock, RAASMC and ECV-304 cells were transduced at a multiplicity of infection (MOI) of 200 or 1000 pfu per cell in the absence or presence of 10 mM sodium butyrate and the percentage of X-Gal positive cells were counted. The results were compared to cells transduced with *lacZ*-adenovirus under identical conditions (Table 1). In agreement with published results, (Condreay *et al*, 35 *supra*) addition of butyrate to cell cultures increased remarkably the expression of the

transgene, especially with baculoviruses. The RAASMC cells seemed to be more susceptible to baculovirus transduction (91% infected at MOI 1000) than ECV-304 cells (21% infected at MOI 1000). Levels of  $\beta$ -galactosidase activity in the RAASMC cells were also measured by a quantitative biochemical assay with *o*-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG). The results were in line with X-Gal staining showing an increase in the transgene expression after butyrate treatment in the baculovirus-transduced cells.

#### *In vitro toxicity*

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay was used to measure the cytotoxicity of virus preparations (Table 2). Neither baculoviruses nor adenoviruses showed any major cytotoxicity to RAASMC cells in the absence of butyrate at MOI 200 or 1000. However, together with butyrate, some cytotoxicity was detected in these cells with baculoviruses at a MOI of 1000. Similar results were obtained with primary WHHL (Watanabe heritable hyperlipidemic) rabbit fibroblasts except that no cytotoxic effects were detected for baculoviruses in the presence of butyrate at a MOI of 1000 (data not shown).

#### *Gene transfer in vivo*

In order to determine whether baculoviruses can be used for *in vivo* gene transfer, NZW rabbits were killed 3, 7 and 14 days after gene transfer. Due to the nuclear targeting of the  $\beta$ -galactosidase expression, intense X-Gal staining was located in the nuclei of the transduced cells. The number of cells positive for  $\beta$ -galactosidase activity was calculated from the baculovirus-transduced ( $1 \times 10^9$  pfu per artery) carotid arteries and was compared with similarly treated adenovirus-transduced arteries. Both viruses mediated delivery of the marker gene to the vessel wall. The numbers of transgene positive cells for baculovirus- and adenovirus-treated arteries at day 3 were  $12 \pm 5$  and  $23 \pm 7$   $\beta$ -galactosidase positive cells/mm<sup>2</sup> of adventitia ( $11 \pm 4$  and  $19 \pm 6$ /mm<sup>2</sup> of the whole artery wall), respectively. At day 7, the corresponding values were  $17 \pm 6$  and  $22 \pm 7$  ( $15 \pm 5$  and  $18 \pm 6$ ). At day 14, the values were  $0.1 \pm 0$  and  $0.2 \pm 0.1$  ( $0.1 \pm 0$  and  $0.1 \pm 0.1$ ). The baculovirus-mediated gene expression was thus transient with a similar efficiency and time pattern as that of the adenovirus-mediated gene transfer. Transgene expression in the arterial wall was also verified with RT-PCR.

The mean value of intima/media ratio (all arteries) was  $0.18 \pm 0.03$  for baculovirus and  $0.12 \pm 0.01$  for adenovirus-treated arteries, which indicates that the procedures did not damage the vessel wall. Histology of the arteries, 7 days after gene transfer, detected no  $\beta$ -galactosidase activity outside adventitia. For both viruses,

macrophage infiltrates and some T cells were detected in the transduced arteries by RAM-11 and MCA-805 immunostainings, respectively. The arterial structure and endothelium remained intact throughout the experiments. Histological findings at all time-points are summarized in Table 3.

5

**Table 1** Transduction of RAASMC and ECV-304 cell lines with baculoviruses and adenoviruses

10	Cell line	MOI	Butyrate (mM)	Positive cells (%)	
				Baculovirus	Adenovirus
15	RAASMC	200	0	1.0 ± 0.3	1.7 ± 0.8
			10	52.5 ± 0.8	42.1 ± 1.1
		1000	0	5.3 ± 1.3	48.0 ± 5.8
			10	90.6 ± 1.6	87.6 ± 2.3
20	ECV-304	200	0	0	44.2 ± 2.9
			10	2.6 ± 1.7	89.4 ± 1.9
		1000	0	0.3 ± 0.3	87.51 ± 4.0
			10	20.8 ± 7.7	97.6 ± 1.4

25 ± s.e.m.

**Table 2** MTT assay for cytotoxicity of baculoviruses and adenoviruses in RAASMC cells

5	MOI	Butyrate (mM)	Baculovirus (%) <sup>*</sup>	Adenovirus (%) <sup>*</sup>
10	200	0	82 ± 3	98 ± 4
		10	78 ± 2	101 ± 2
	1000	0	81 ± 1	99 ± 1
		10	65 ± 1	90 ± 3

15 \* Survival percentage of RAASMC cells as compared with mock-transduced cells.  
± s.e.m.

**Table 3** Summary of histological findings in transduced arteries 3, 7 and 14 days after  
20 gene transfer

25	Time-point	Virus	Macrophages	PMN cells	Endothelium intact
30	3d	adeno	++	+	Yes
		baculo	++	++	Yes
	7d	adeno	+	+	Yes
		baculo	+	+	Yes
35	14 d	adeno	++	-	Yes
		baculo	++	-	Yes

-, absent; + mild; ++ moderate and +++ severe infiltrates as compared with control arteries.

PMN, polymorphonuclear.

**Example 2**

Using essentially the same procedure as in Example 1, it has been shown that baculovirus gene transfer works also in brain and skeletal muscle. Using baculovirus//*lacZ*, rat brain shows positive transfection in various types of brain cells, 5 especially in choroid plexus cells in ventricles and endothelial cells. The profile of transfected cells is clearly different from that of adenoviruses.

Further, baculovirus transfection has been demonstrated in rabbit skeletal muscle. Baculovirus encoding *lacZ* ( $1.8 \times 10^{10}$  PFU) was directly injected into the adductor muscle of NZW rabbit *via* a 25 G needle. The injection volume was 0.5 ml. 10 Tissue samples were collected 7 days after the gene transfer, and X-Gal staining was performed overnight. These results clearly indicate that baculovirus can be used for transfection of several cell types in mammals, i.e. not only arterial cells.

The accompanying drawing illustrates the construction of a nuclear-targeted  $\beta$ -galactosidase-encoding baculovirus transfection cassette. In principle, this is a 15 standard public domain baculovirus with polyhedrin promoter, into which have been cloned restriction sites and the CMV-NT *lacZ* expression cassette. The *lacZ* expression cassette is oriented opposite to the polyhedrin promoter. The sequence of the CMV-nt *lacZ* expression cassette is in SEQ ID NO:7.

**CONSTRUCTION OF NUCLEAR TARGETED  $\beta$ -GALACTOSIDASE ENCODING BACULOVIRUS**

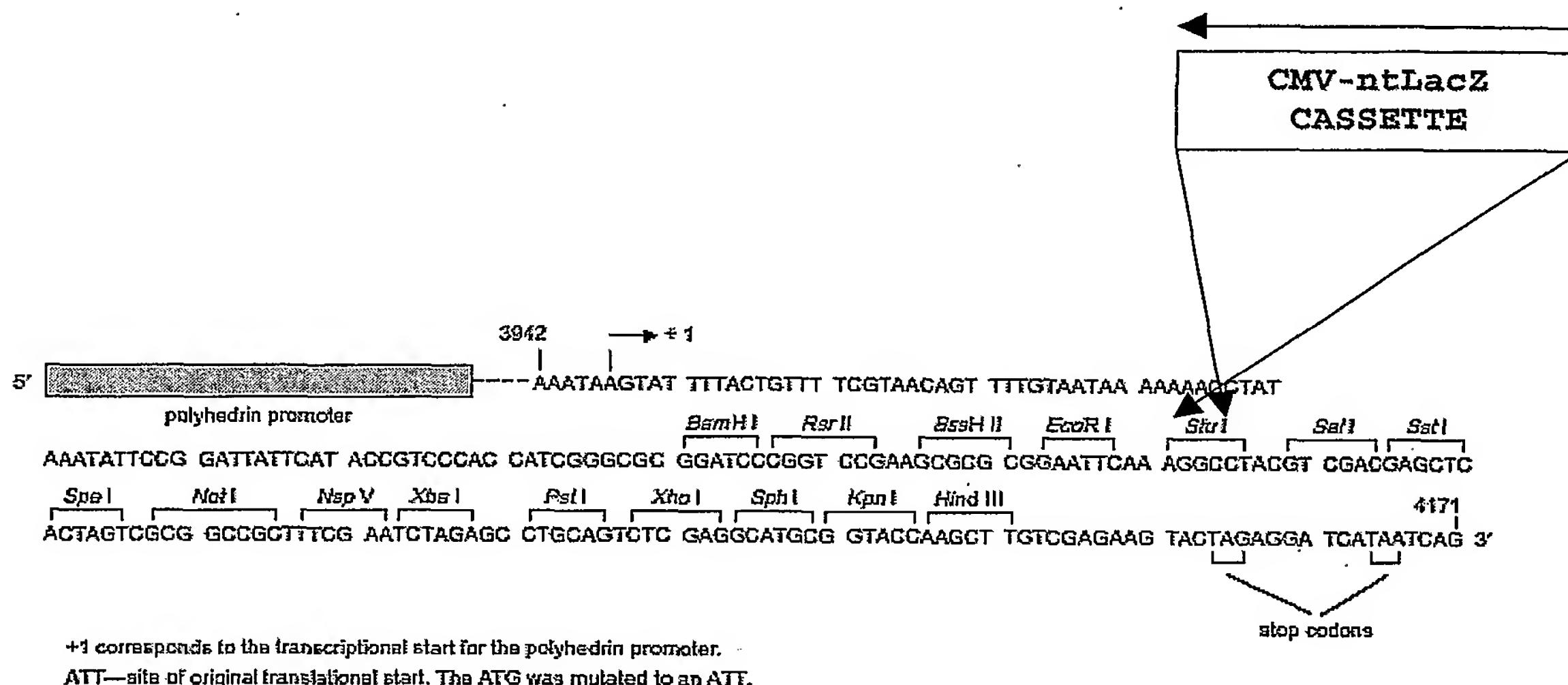


FIGURE 1

## SEQUENCE LISTING

<110> Ark Therapeutics Ltd.

<120> GENE DELIVERY OF A VIRAL VECTOR

<130> REP06446WO

<140> not yet known

<141> 2001-05-29

<160> 7

<170> PatentIn Ver. 2.1

<210> 1

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
Oligonucleotide

<400> 1

ttggaggcct aggctttgc

20

<210> 2

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
Oligonucleotide

<400> 2

ttggcctaga gtcgacggat

20

<210> 3

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

## Oligonucleotide

&lt;400&gt; 3

tgaggggacg acgacagtat

20

&lt;210&gt; 4

&lt;211&gt; 22

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:

Oligonucleotide

&lt;400&gt; 4

ggtagaagac cccaaaggact tt

22

&lt;210&gt; 5

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:

Oligonucleotide

&lt;400&gt; 5

ccaagaagaa acgcaaagtg

20

&lt;210&gt; 6

&lt;211&gt; 17

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:

Oligonucleotide

&lt;400&gt; 6

cgccattcgc cattcag

17

&lt;210&gt; 7

&lt;211&gt; 4495

&lt;212&gt; DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Recombinant  
baculovirus

<400> 7

ccattgcata cgttgtatct atatcataat atgtacattt atattggctc atgtccaata 60  
tgaccgccat gttgacattg attattgact agttattaaat agtaatcaat tacggggtca 120  
ttagttcata gcccataatggagttccgc gttacataac ttacggtaaa tggcccgct 180  
ggctgaccgc ccaacgaccc cggccattg acgtcaataa tgacgtatgt tcccatagta 240  
acgccaatag ggactttcca ttgacgtcaa tgggtggagt atttacggta aactgcccac 300  
ttggcagttac atcaagtgtatcatatgcca agtccgcccc ctattgacgt caatgacggt 360  
aaatggcccg cctggcatta tgcccagttac atgaccttac gggactttcc tacttggcag 420  
tacatctacg tattagtcat cgctattacc atgggtatgc ggtttggca gtacaccaat 480  
ggcggtggat agcggtttga ctcacgggaa tttccaagtc tccacccat tgacgtcaat 540  
gggagtttgt tttggcacca aaatcaacgg gactttccaa aatgtcgtaa taacccggcc 600  
ccgttgacgc aaatggcggtt taggcgtgtatcggtggagg tctatataag cagagctcg 660  
ttagtgaacc gtcagatctc tagaagcttg gcctagagtc gacggatccg gggattccc 720  
cagtctcagg atccaccatg gggcccaaga agaaacgcaa agtggggagc atgggggatc 780  
ccgtcggttt acaacgtcgactgtggaaa accctggcgtaa tacccaaatc aatcgcttg 840  
cagcacatcc ccctttccca gctggcgtaa tagcgaagag gcccgcacccg atcgcccttc 900  
ccaacagttg cgcaacgtcgactgtggaaatg ggcgtttgcc tggtttcgca ccagaagcg 960  
tgccggaaag ctggctggag tgcgatctc ctgaggccga tactgtcgatcg 1020  
actggcagat gcacggttac gatgcggccatc tctacacccaa cgtaacccat cccattacgg 1080  
tcaatccgcc gtttgttccc acggagaatc cgacgggttg ttactcgctc acatttaatg 1140  
ttgatgaaag ctggctacag gaaggccaga cgcaattat ttttgcgttgc gttactcg 1200  
cggttcatct gtggcgcaac gggcgctggg tcgggttacgg ccaggacagt cggttgcgt 1260  
ctgaatttgc cctgagcgca ttttgcgttgc ccggagaaaa ccgcctcgcc gtgtatggc 1320  
tgcgttggag tgacggcgt tatctggaaatc caggatat gtggcgatg agcggttgc 1380  
tccgtgacgt ctcgttgcgt cataaaccga ctacacaaat cagcgatttc catgttgc 1440  
ctcgctttaa tgatgatttc agccgcgtg tactggaggc tgaagttcag atgtgcggcg 1500  
agttgcgtga ctacctacgg gtaacagttt ctatggca gggtaaaacg caggtcgcca 1560  
gcggcaccgc gcctttcgcc ggtgaaatttc tcgatgagcg tggtggatgc ggcgtcg 1620  
tcacactacg tctgaacgtc gaaaacccga aactgtggag cgccgaaatc ccgaatctct 1680  
atcgtgcgggt ggtgaactg cacaccgcg acggcacgtt gattgaagca gaagcctgc 1740  
atgtcggtt ccgcgagggtt cggattgaaa atggctgtgc gctgctgaac ggcaagccgt 1800  
tgctgattcg aggcgttaac cgtcacgagc atcatcctct gcatggcgtatgc gtcatggatg 1860  
agcagacgt ggtgcaggat atcctgtgc tgaagcagaa caactttaac ggcgtgcgt 1920  
gttcgcattt tccgaaccat ccgctgtggt acacgctgtg cgaccgtac ggcctgtatg 1980  
tggtgatgc agccaatatt gaaacccacg gcatggcgcc aatgaatcgt ctgaccgtatgc 2040  
atccgcgtcg gctaccggcg atgagcgaac gcgtaacgcg aatgggtgcag cgcgatcgta 2100  
atcaccgcgag tgtgatcattc tggtcgctgg ggaatgaatc aggccacggc gctaatcagc 2160  
acgcgtgttgc tgcgtggatc aaatctgtcg atccttcccg cccgggtgcag tatgaaggcg 2220  
gcggagccga caccacggcc accgatatttttgcgttgc gtcgcgcgt gtggatgaaag 2280  
accagccctt cccggctgttgc cggaaatggatccatcaaaaa atggctttcg ctacctggag 2340  
agacgcgcggcc gctgatcattt tgcgtatgc cccacgcgtatgc ggttaacagt cttggcggtt 2400  
tcgctaaata ctggcaggcg tttcgatcgt atccccgttt acagggcgcc ttcgtctggg 2460

actgggtgga tcagtcgctg attaaatatg atgaaaacgg caacccgtgg tcggcttacg 2520  
gcgggtgattt tggcgatacg ccgaacgatc gccagttctg tatgaacggc ctggctttg 2580  
ccgaccgcac gccgcaccca gcgctgacgg aagcaaaaca ccagcagca gttttccagt 2640  
tccgtttatc cgggcaaacc atcgaagtga ccagcgaata cctgttccgt catagcgata 2700  
acgagctcct gcactggatg gtggcgctgg atggttaagcc gctggcaagc ggtgaagtgc 2760  
ctctggatgt cgctccacaa ggtaaacagt tgattgaact gcctgaacta ccgcagccgg 2820  
agagcgccgg gcaactctgg ctcacagtac gcgttagtgca accgaacgac accgcattgt 2880  
cagaagccgg gcacatcagc gcctggcagc agtggcgtct ggccggaaac ctcagtgtga 2940  
cgctccccgc cgctcccac gccatcccgc atctgaccac cagcgaaatg gatTTTgca 3000  
tcgagctggg taataagcgt tggcaattta accgccagtc aggctttctt tcacagatgt 3060  
ggattggcga taaaaaaacaa ctgctgacgc cgctgcgcga tcagttcacc cgtgcaccgc 3120  
tggataacga cattggcgta agtgaagcga cccgcattga ccctaacgac tgggtcgaac 3180  
gctggaaggc ggcgggcat taccaggccg aagcagcgtt gttgcagtgc acggcagata 3240  
cacttgctga tgcggtgctg attacgaccg ctcacgcgtg gcagcatcag gggaaaacct 3300  
tatttatcag ccggaaaacc taccggattt atggtagtgg tcaaattggcg attaccgtt 3360  
atgttgaagt ggcgagcgt acaccgcac cggcgcggat tggctgaac tgccagctgg 3420  
cgcaggttagc agagcgggta aactggctcg gattaggcc gcaagaaaac tatcccacc 3480  
gccttactgc cgctgtttt gaccgctggg atctgccatt gtcagacatg tataccccgt 3540  
acgtcttccc gagcgaaaac ggtctgcgtc gcgggacgcg cgaattgaat tatggcccac 3600  
accagtggcg cggcgacttc cagttcaaca tcagccgcta cagtcacacg caactgatgg 3660  
aaaccagcca tcgccatctg ctgcacgcgg aagaaggcac atggctgaat atcgacggtt 3720  
tccatatggg gattggtggc gacgactcct ggagccggtc agtacggcg gaattccagc 3780  
tgagcgccgg tcgctaccat taccagttgg tctgggtgtca aaaataataa taaccggca 3840  
gggggatcgc agatcggcca gataccgatg ctgccgcagc aaaaagcagg agcagatgcc 3900  
gccgtcgccag saaagatgtc gcasaggagg aggcgatgtc gccggcggag gaggcgaagt 3960  
aagtagaggg ctgggctggg ctgtgggggg gtgtgggggtc cgggactggg cagtctggg 4020  
gtccctctca ccactttct taccttcta ggatgctgcc gtgcggccg ctcatacacc 4080  
ataaggttaa aaaaatacta gatgcacaga atagcaagtc catcaaaaact cctgcgtgag 4140  
aattttacca gacttcaaga gcatctgcc acatcttcaa aaatgccacc gtccgatgaa 4200  
aaacaggagc ctgctaagaa acaatgccac ctgtcaataa atgttgaaaa ctcatccat 4260  
tcctgcctct tggtccttgg gcttggggag gggtgccgcgg atgtggtag ggaacatgac 4320  
tggtaaatg ggaaggcgtt caaaagaatt cccaatattg actaccaagc cacctgtaca 4380  
gatctgcctg catgctttgc atacttctgc ctgctggggc gcctggggac tttccacacc 4440  
ctaactgaca cacattccac agccggatct gaggaacccc tagtgtatgga gttgg 4495

## INTERNATIONAL SEARCH REPORT

Inte      nal Application No

PCT/GB 01/02383

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 C12N15/86 A61K48/00 A61P9/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, WPI Data, PAJ, EPO-Internal, MEDLINE, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category <sup>°</sup>	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 20027 A (YLAE HERTTUALA SEppo ;MARTIN JOHN FRANCIS (GB); BARKER STEPHEN GEO) 14 May 1998 (1998-05-14) cited in the application page 1, line 4 - line 7 page 4, line 13 -page 6, line 22 page 12, line 16 - line 29 page 16, line 4 - line 23 ---	1-5,8-11
X	WO 00 05394 A (SARKIS CHAMSY ;MALLET JACQUES (FR); RHONE POULENC RORER SA (FR)) 3 February 2000 (2000-02-03) cited in the application page 1, line 4 - line 9 page 3, line 20 -page 4, line 14 page 24 -page 27; examples 4,5 ---	1,6,8
		-/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## ° Special categories of cited documents :

- °A° document defining the general state of the art which is not considered to be of particular relevance
- °E° earlier document but published on or after the international filing date
- °L° document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- °O° document referring to an oral disclosure, use, exhibition or other means
- °P° document published prior to the international filing date but later than the priority date claimed

- °T° later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

- °X° document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

- °Y° document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

- °&° document member of the same patent family

Date of the actual completion of the international search

21 August 2001

Date of mailing of the international search report

04/09/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

## INTERNATIONAL SEARCH REPORT

International Application No

PL, GB 01/02383

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1998 MCMAHON J M ET AL: "Inflammatory responses following direct injection of plasmid DNA into skeletal muscle." Database accession no. PREV199800479471 XP002175450 abstract &amp; GENE THERAPY, vol. 5, no. 9, 1998, pages 1283-1290, ISSN: 0969-7128</p> <p>---</p>	7
A	<p>DE 197 35 593 A (HEPAVEC AG FUER GENTHERAPIE) 18 February 1999 (1999-02-18) column 1, paragraph 1 -column 2, paragraph 2; example 2</p> <p>---</p>	1
A	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; April 1998 (1998-04) HOFMANN C ET AL: "Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system." Database accession no. PREV199800256742 XP002175451 abstract &amp; GENE THERAPY, vol. 5, no. 4, April 1998 (1998-04), pages 531-536, ISSN: 0969-7128</p> <p>---</p>	1
A	<p>SANDIG V ET AL: "GENE TRANSFER INTO HEPATOCYTES AND HUMAN TISSUE BY BACULOVIRUS VECTORS" HUMAN GENE THERAPY, XX, XX, vol. 7, no. 16, 20 October 1996 (1996-10-20), pages 1937-1945, XP000674903 ISSN: 1043-0342 cited in the application page 1937 abstract page 1943, paragraph 4</p> <p>---</p> <p>-/-</p>	1

## INTERNATIONAL SEARCH REPORT

International Application No

PCT, GB 01/02383

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 20 January 1999 (1999-01-20) LO WARREN D ET AL: "Adeno-associated virus-mediated gene transfer to the brain: Duration and modulation of expression." Database accession no. PREV199900119131 XP002175452 abstract &amp; HUMAN GENE THERAPY, vol. 10, no. 2, 20 January 1999 (1999-01-20), pages 201-213, ISSN: 1043-0342</p> <p>---</p>	6
P, X	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 20 May 2001 (2001-05-20) PIERONI LUISA ET AL: "In vivo gene transfer in mouse skeletal muscle mediated by baculovirus vectors." Database accession no. PREV200100278102 XP002175453 abstract &amp; HUMAN GENE THERAPY, vol. 12, no. 8, 20 May 2001 (2001-05-20), pages 871-881, ISSN: 1043-0342</p> <p>---</p>	1,7,8
P, X	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; September 2000 (2000-09) AIRENNE K J ET AL: "Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery." Database accession no. PREV200000439639 XP002175454 abstract &amp; GENE THERAPY, vol. 7, no. 17, September 2000 (2000-09), pages 1499-1504, ISSN: 0969-7128</p> <p>-----</p>	1,2,5, 8-10

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PL, GB 01/02383

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
WO 9820027	A 14-05-1998	AU	729420	B	01-02-2001
		AU	4790697	A	29-05-1998
		EP	0941116	A	15-09-1999
		JP	2001503755	T	21-03-2001
		NO	992106	A	30-06-1999
		PL	333272	A	22-11-1999
WO 0005394	A 03-02-2000	FR	2781503	A	28-01-2000
		AU	4916299	A	14-02-2000
		EP	1100946	A	23-05-2001
DE 19735593	A 18-02-1999	WO	9909193	A	25-02-1999
		EP	1003896	A	31-05-2000
		ES	2150894	T	16-12-2000